PHYSIOLOGY OF THE RESPIRATORY SYSTEM

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# THE COUNCIL FOR TOBACCO RESEARCH - U.S.A.

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Application For Research Grant

Date: January 17, 1966

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4. Project or Subject:

The Development and Biology of Lung Surfactant (Year IV).

5. Detailed Plan of Procedure (Use additional pages if more space is required.)

Many laboratories in this country are considering lung surfactant as their major interest; nonetheless, in the last calendar year, there has been little advance in sound working information about synthesis and turnover of this interesting phospholipid. Harlan's investigation in dogs with C-14 labeled palmitate linked up the production of surfactant with lung phospholipid turnover, and demonstrated that the half-life of palmitate label was shortened by the experimental production of pulmonary edema (9). Pursuing a different approach, our group made two observations: 1) the appearance of both acetate and palmitate into phospholipid moieties of lungs is incredibly rapid, especially with acetate precursor! 2) this anabolism is associated with the large alveolar (septal) cells exclusively, as radioautographs showed. (1) Taken together, these two investigations inferentially link dipalmityl lecithin formation with lung surfactant production, and underline the truly incredible metabolic machinery contained in these cells. Macklin, of course, termed the septal cells "a diffuse organ within an organ". (10) Unpublished data obtained by the Yale*(Gluck) and Morgan (Washington) have begun to dissect the metabolic pathways of lung phospholipid synthesis. These represent the first efforts to outline enzymes that may be involved in synthesis of this disaturated phosphatide. There appears to be a definite relationship between phosphatidylethanolamine and phosphotidylcholine pathways in lung. Morgan thinks two specific transferases are involved and is isolating them. Felts (California) continues to investigate lipid handling by lung, including the fashion in which precursors are extracted from the circulation. (11)

* group

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Beyond finding that phospholipid labeled with tritiated acetate occupies a geographic position in the alveolar cell cytoplasm other than the special inclusion bodies -- which indicates to us that PL synthesis may occur outside of these organelles -- we know of no data to further clarify the relationship of the special inclusion body to surfactant phospholipid formation. It is hoped that electron microscopic autoradiography of lipid soluble material will be more satisfactory in the future. Following our report that the inclusions were alkaline phosphatase positive (indicating their non-mitochondrial nature) (2), Canadian workers described in newborn lungs a cytoplasmic lamellar body from which they thought the inclusions developed. The "precursor granule" was acid phosphatase positive. This might indicate one of two possibilities: 1) early secretory granules coming off Golgi (which are acid phosphatase positive, according to Novikoff). The author's interpretation, however, was that the material was 2) a degenerative autophagic inclusion of some sort, perhaps related to the lysosome. (12) We, and also Campiche (13), have now identified these precursor bodies morphologically. Our failure to obtain acid phosphatase activity in the mature inclusions corresponded to their own findings.

It does seem, however, that most of the important biological questions about the alveolar cell remain elusive. Among the major unanswered questions are the following: 1) its derivation, whether entodermal or mesodermal; 2) its relationship, if any, to the alveolar macrophage; 3) the biological role and identity of the alveolar cell inclusion bodies; 4) the answer to the question Macklin asked regarding the mucoid nature of the alveolar lining substance; 5) information regarding any controlling mechanisms of the production or inhibition of surfactant phospholipid, either inside or outside the cell; 6) whether pulmonary edema develops from damage to the cell or its product, or occurs the other way around, or both; 7) whether its inhibition in immature lungs is a reflection of failure of production, functional depletion, or inhibition by, say, exogenous protein-containing materials in the alveolar space.

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If experience continues to repeat itself, some projects in our investigation will move rapidly toward a successful conclusion, while others are gestating various technical difficulties. For this reason, several aspects of a general question will be in some stage of prosecution at any given time.

The work being outlinedwill itself constitute a kind of progress report, since each study has progressed some (variable) distance toward publication. Those mentioned first have accumulated the most data.

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1. The pathogenesis of stress-induced pulmonary lesions leading to surfactant inhibition: high oxygen pressure (HOP). (Buckingham, Sommers, McNary)

We assume, but do not know, that the large alveolar cell is generically trophic, i.e., it will respond to nervous stimuli or to neurohumors released in the immediate vicinity. We previously suggested (3) that the pathogenesis of the alveolar hyaline membrane in infancy might involve "hypersecretion" of the respiratory epithelium. When Jamieson and van den Brenk (14) reported surfactant inhibition by high oxygen pressure in rats that was modified by the action of barbiturates, we considered that HOP pulmonary edema might result from direct neurogenic stimulation of the surfactant producing alveolar cell. Knowing that some systemic stresses deplete catecholamines in the brainstem (presumably by increasing the functional activity of sympathetic neurons), it seemed theoretically possible that such a situation might obtain in HOP and, moreover, that prolonged central discharges might lower neurotransmitter (NE) stores in peripheral organs, including the heart and lung, and thus alter the normal output of lung surfactant. This particular study was high in priority because high oxygen pressure had been suggested as a treatment of respiratory distress syndrome. Even at the outset we felt this treatment to be contraindicated, inasmuch as we suspect central autonomic centers are tentative in these infants, and might suffer unduly from an agent known to be a central nervous system toxin.

The data obtained to date, which have been reported at the Columbia Medical Society (December 1965) and will be discussed at Federation meetings (4), have approached the question of the pathogenesis of the lung lesions by way of their central nervous system connections. A theoretical framework for stress was established by Vogt (15) and also by Maynert (16).

Adult rats exposed to 75 psi gauge pressure (5 atm) for 30 minutes were found to lose more than 50% of their brainstem norepinephrine, and somewhat more than that from the myocardium (65%). After only 15 minutes (an exposure that produces no clinical symptoms in animals), 25% of the brain catecholamines are already lost. The stress-induced loss of NE in both the brain and heart was completely prevented by pretreatment with 75 mg/K of pentobarbital.

Furthermore, lung weights of barbiturate-HOP animals remained normal, and lesions to be described did not develop. Barbiturates are known to interfere with the reticular activating system, and its mode of action here may be to inhibit afferent impulses to the hypothalamus. Pentobarbital also interferes with recovery time of autonomic ganglia, suggesting a second mechanism. (8)

It was not feasible to measure norepinephrine depletion in the lung, inasmuch as tissue concentrations are too low to assess changes with reliability. We plan to study lung NE stores by means of fluorescence microscopy (Falck). (17)

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We were interested to study the effects of HOP on lung serotonin (5 HT) for several reasons: rodents store great quantities of this amine in their lungs, and have been suggested as a "serotonin reactive lung species". (18) Unlike guinea pigs, whose lungs contain a high concentration of histamine and to which amine they react, rats and mice tolerate injected histamine that would be many times over fatal to guinea pigs or to man. (19) However, rodents respond to serotonin. Serotonin is a mediator, furthermore, and is a very potent pulmonary hypertensive agent in all species studied. Its role as a mediator has been suggested in endotoxin administration (20) and in the pulmonary hypertension associated with experimental microemboli. (21)

In our experiments (5 atm HOP 30 min), lung serotonin content in rats was reduced to 50% of its normal levels. As with the catecholamine stores, barbiturate premedication somewhat prevented these 5 HT losses in lung, but was not completely protective. We have identified the cellular site of serotonin storage in rat lung by means of autoradiography following intravenous administration of tritiated 5 HT (5). As in other viscera, mast cells of rat lung selectively accumulate grains. Using other methods, Rowley and Benditt had previously found that serotonin in rat skin was partially contained in mast cells. (22) Mast cells are very abundant in rat lung and are, for the most part, located in the adventitia of small vessels.

When HOP-treated lungs were stained with Giemsa to identify mast cells, it was apparent that lung serotonin release must involve losses from endogenous mast cell stores, inasmuch as these elements were often almost completely degranulated. In contrast, the lung mast cells of barbiturate-protected HOP animals contained abundant numerous healthy granulated mast cells.

Another source of the serotonin released in lung might be from circulating trapped platelets (vide infra).

Lungs were examined by light and electron microscopy following various dosages of HOP, ranging from 45 to 75 psi pressure, along with barbiturate-treated HOP, and with normal control lungs. The primary lesion in the lungs of HOP animals occurred in the smallest pulmonary venules. This was first seen in a 45 psi animal and, to a modest degreee, in a 75 psi pentobarbital-treated HOP animal. The lesion consisted of palisading of white cells and platelets onto the endothelial surface, hyaline degeneration of the venular wall, and the collection of fluid and cells in the perivenous lymphatics. This clearly indicates that this may be the primary site of high oxygen pressure pulmonary injury, and cells in the lymphatics indicate selective leakage at this point. This is of great interest, because Majno and Palade (23) have found that several mediators of vascular permeability, including serotonin, bradykinin, and histamine, select the post-capillary venule in other organs as the primary site of leakage, and Rowley has forwarded evidence to suggest that smooth muscle contraction precedes production of the leak. (24)

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Whether released serotonin from mast cells is a mediator in the venular lesion must remain speculative. It is perhaps more likely that an interaction on the venular surface between endothelium and the platelets/WBC may stimulate the release of factors that aggravate inflammation, one or more of which may act to degranulate the mast cell granules. We do not suggest that mast cell 5 HT depletion occurs by way of a neurogenic impulse. Uvnäs believes that histamine release from mast cells by small molecular weight releasers, such as 48/80, unmasks a phospholipase A contained in the mast cell membrane which then itself releases the amines. (25)

It is not known whether 5 HT release from undamaged platelets occurs in vivo but, in vitro, quantitative release of serotonin can be brought about by very low outside concentrations of either trypsin or thrombin (6), but by no other enzymes studied. (7) Furthermore, amine release does not affect the morphology or the metabolic efficiency of the platelets. Generation of tiny amounts of thrombin during the trapping of platelets in the venular and capillary walls of the lung following exposure to HDP can be inferred from examination of other small vessels in which blobs of profibrin were present. It is possible that this mechanism could account for release of small amounts of platelet serotonin into the vessel wall and instigate subsequent lesion and leakage from the venule. Although histamine content was not studied, its activity as a mediator in this situation is less likely -- even though it is present in mast cell granules of the rat -- because of the great tolerance rodents have for histamine.

In the severely damaged edematous HDP lung, a series of vascular changes, ranging from venular damage to platelet and fibrin thrombi in capillaries, venular and periarterial edema, congestion, and even exudation of fibrin into the alveolar walls through damaged alveolar capillaries and venules, were present in various parts of an individual lung. The large alveolar cells were normal-appearing except for one bizarre feature, namely, the development of a large cytoplasmic bleb on the air face that was filled with endoplasmic-like structures. Such a lesion has not been previously described. Microvilli were present on the air surface of alveolar cells, and the numbers of cytoplasmic inclusions were reasonably normal.

The surface activity of high oxygen pressure lung extracts was tested on the Wilhelmy ballance and, after 30 minutes (75 psi), all lung extracts were normal. Even after 1 hr., two/seven extracts retained their normal surface activity. Thus, we must conclude that surfactant inhibition in this situation is probably not a direct effect on the alveolar cell, but may occur secondary to the leakage of fibrinous exudate into the alveolar space that could form even a coacervate with the surface active lipoprotein. (26)

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Bean (27) hypothesized that the sympathetic nervous system was somehow involved with the toxicity of high oxygen pressure since adrenal medullectomy, as well as chlorpromazine pretreatment, were protective. However, these are the first data that speak directly to the Bean hypothesis and they support it. Furthermore, they indicate that peripheral effects of what we presume is a CNS discharge include the myocardial catecholamines ( and ?lung), and even epinephrine secretion from the adrenal medulla. The effects of increased circulating catecholamines may help to exacerbate the venular lesion in the lung.

We will also study with Dr. T. Omura whether abnormality in the electrical impulse of the myocardium results from discharge of NE granules from the cardiac nerves in the heart. We plan to examine the amine depletion in this stress system by means of fluorescence microscopy, as well as by chemical analysis. This will permit the localization of stores in affected nerves.

II. The mechanism of pulmonary edema and surfactant alteration induced by vagotomy. (Buckingham, Sommers, Goldenberg, McNary) (8)

Klaus et al (28) in 1961 reported that bilateral vagotomy destroyed normal surface activity of lung extract, and reduced the number of inclusions in the large alveolar cells (4-6 hrs.). Decreased numbers of inclusions that corresponded in time with surfactant alteration also came from exposure to $CO_2$ intoxication (24-48 hrs.). (29) Surfactant alteration occurs 1 hr. after high oxygen pressure; this makes HOP the most rapid experimental means to produce experimental alteration of surfactant in adult animals.

Vagotomy-induced edema remains of interest for those concerned with the pathogenesis of hyaline membrane disease, because Miller reported that hyaline membranes develop in bilaterally vagotomized animals. (30) We also have found a lesion in the nucleus of the vagus nerve in the brainstems of these infants, as well as in some with fetal pulmonary hemorrhage. (9)

Cervical section of the vagus nerves produces widespread changes, of course, not only in efferent autonomic control but disturbs afferent stimuli, many visceral fibers of which travel in the vagus nerve. Numerous studies of the mechanism of pulmonary edema formation in this circumstance have reached divergent or inconclusive opinions. Some workers doubt that parasympathetic nerves are even present in pulmonary vessels, although Hebb (31) has demonstrated cholinesterase in small pulmonary arterial ganglia that must be parasympathetic (since sympathetic ganglia are all located in the paravertebral region). Kraus feels from in vivo studies, utilizing thoracic windows, that the vagus controls the pattern of intermittent flow observed to involve entire lung sublobules at normal lung volumes. (32) Farber, earlier, concluded that parasympathetic nerves to the pulmonary vessels might be vasoconstrictors. (33)

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At any rate, the final effect of vagotomy would be to increase sympathetic influences on the lung, and on the heart. Thus, vagotomy may resemble in some respects the HOP situation previously discussed in which prolonged central sympathetic discharge may set the stage for high oxygen pressure pulmonary edema.

We are applying the technique of Majno and Palade (23) to outline the sequence of pathogenetic lesions following vagotomy, at intervals from one-half to six hours after an operative procedure. Pelikan Ink is injected into the tail vein 5 minutes before sacrifice of each animal. This may afford a demonstration of the intimate vascular site in the lung where plasma leakage first occurs. Complete series of animals have been prepared for examination by light and electron microscopy. In addition, lung surface extracts of vagotomized animals have been studied on the Wilhelmy balance, and specimens of lungs showing altered extract have been prepared by the method of Laznitzky to observe whether the normal alveolar membrane disappears at the time that surfactant is altered. Detailed information from this material will be available in a few weeks; preliminary micrographs suggest that, following vagotomy, both the vascular endothelium and the alveolar epithelium may be simultaneously involved. Microvilli disappear from the air surface of alveolar cells, so that the cells resemble macrophages in the septal spaces. Membranous alveolar epithelium is thickened, and an active inflammatory response proceeds in the pulmonary capillaries. Cursory examination of the ink-injected lungs indicates that the leakage appears at the capillary level, but this requires much more study.

III. Antigenicity of dipalmityl lecithin (DPL) in rabbits. (Craig, Buckingham, Rothenberg, McNary)

Craig has localized fluorescent-tagged antibody to washed human lung foam (DPL containing protein) in human lungs. He indicates that, in fetal lung, antibody appears in the differentiating alveolar cell before the time it is observed at the alveolar interface as the normal lung lining layer. Of great interest is his finding that the abnormal alveolar hyaline membranes of infant lungs contain huge amounts of fluorescent-tagged material indicating, as previously suggested (3), that bronchiolar and alveolar cell necrobiosis was a prominent part of the infant disease in which contents of epithelial cell were incorporated into the membrane. Craig further suggests that lung lining layer already secreted may find its way into the membrane, thus accounting for the continuing progressive atelectasis that appears to characterize this illness. (34)

We discussed his antigen with Craig in terms of its non-specificity (obtained as it was from lung foam which, despite many washings, contains various lung proteins, some probably derived even from plasma). It was our hope that he might obtain even more significant data if an antigen, resembling the surface active moiety of lung foam, were used to stimulate antibody production. We first thought of trying dipalmityl lecithin, now available in synthetic form. This has been mixed with Freund's adjuvant and injected in rabbits at weekly intervals x4. Trial bleeding ten days after the last injection has failed so far to demonstrate any antibody, as measured on an Ouchterlony plate. The rabbits are being rested, and a series of antigen injections will be reinstituted for six weeks more.

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A trial of antibody formation will also be tested in sheep (whose antibody production is far superior to other mammals), using this trial antigen as a first approximation. Recovery of any antibody will also facilitate the study of the localization of ferritin labeled antibody in alveolar cells by electron microscopy (McNary).

If this phosphatide is not antigenic, we will discuss with an organic chemist the possibility of conjugating the DPL molecule to increase its antigenicity.

Craig's data are so interesting, it is too bad that the non-specificity of his antigen may vitiate his elegant findings.

IV. Entodermal origin of large pulmonary alveolar cells. (Volkmann and Buckingham)

Neither the site nor the germ layer of origin of the large alveolar (septal) cells is certain.(35) All the evidence obtained to date in our laboratory in re the morphologic differentiation of the fetal alveolar elements suggests an epithelial cell origin. Early in development, the epithelial cells resemble each other, later taking on morphologic characteristics of septal and membranous cells. Furthermore, we feel that the "lung-like" phospholipid synthetic activity that we have now localized specifically to the large alveolar cell (1) indirectly speaks to its origin, putting it at least in the lung. This makes it unlikely that even if the cell is mesodermal its precursors arise from bone marrow, as do peritoneal macrophages. (36)

These studies will use C-3 H-black inbred mice (that can tolerate parabiosis). We have induced the multiplication of pulmonary alveolar wall cells and macrophages by intravenous administration of BCG, noting the increase in the cells of the alveolus 24 and 48 hours after injection. We will study the latent period of recovery of tritiated thymidine label in the harvest of alveolar cells and in lungs of animals sacrificed for periods up to 12 to 16 hours after I.V. label injection. Considerable appearance of label at this time diminishes the possibility of a distant mesodermal origin. This can be made more convincing in parabionts.

These experiments will be to the stage of thymidine injection by February 1966. The survival of parabionts is a technically limiting factor at the present time.

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V. Manufacture of acid mucopolysaccharide in large alveolar cell inclusions. (Buckingham and McNary)

We think it is possible and relevant to understanding the integrity of the alveolar membrane that mucoid material underlies the surface active phospholipid in the pulmonary alveolar lining layer. This would correspond with Macklin's original suggestion. (37) Confirmation of the layer's PAS-positive, diastase-resistant staining properties at electron microscopic magnifications(38) further strengthens Macklin's claim. We have found that some cytoplasmic inclusions in the alveolar cells contain PAS-positive, diastase-resistant material. (1) (2) Interest in the nature and function of the surface active material, which we know to be phospholipid, has apparently diminished curiosity about what else is in the membrane. Its estimated thickness (200 to 3,000 Angstrom units) suggests a complex substance, and lipid + protein is not PAS-positive. Therefore, Macklin as usual is probably right. He supposed the function of this layer -- which he termed 'mucoid' -- was to preserve the interface from desiccation. One might also imagine that penetration of microörganisms through a jelly-like substance would make alveolar cell invasion and entry into the blood stream somewhat more difficult. Therefore, the possible role of the hydrated mucoid lining in the layer is certainly relevant to the biology of the alveolus. One could speculate that if such a layer altered its sol-gel state, as does connective tissue ground substance, great differences in permeability of the alveolar membrane might result.

What would be the nature of the mucoid? Since hyaluonic acid is non-sulfated, it does not take a PAS-positive, diastase-resistant stain. One is left with the probability of a sulfated mucoid, such as heparin or mucoitin sulphuric acid.

Screening experiments will study the radioautographic localization of S-35 in mice during a 24-hr. period. Localization in alveolar cell or macrophage cytoplasm and the time that this occurs can be estimated sufficiently that, in further experiments, electron microscopic radioautographs can be prepared by the method of Hay. Localization will be studied in comparison to that in proximal bronchiolar epithelium, as well as in the small intestinal epithelial cells of mice; these other tissues serving as controls.

VI. Precocious induction of fetal surfactant function. (Buckingham, Adamson, and McNary)

Since outlining this project in last year's grant application, we had an opportunity to discuss the project with Dr. Florence Moog, the zoölogist who discovered gut epithelium in developing the relationship between alkaline phosphatase activity and functional differentiation. (39) She encouraged us to prosecute the analogy in lung with experimental evidence. Fetal lung and fetal gut may be analogous tissues (both developing from entoderm), and may therefore be susceptible to some of these same fetal influences. Moog found that glycogen depletion and alkaline phosphatase enzyme activity increase heralded the morphological and functional differentiation of fetal gut; furthermore, the activity could be precociously induced by means of ACTH in some but not all species.

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In differentiating fetal pulmonary alveolar epithelium, glycogen storage is also abundant (2) (40) until just prior to the time that surfactant is first measurable in fetal lung; moreover, the inclusions that we rightly or wrongly associate with surfactant formation are alkaline phosphatase positive (2). Therefore, it is reasonable to wonder whether differentiation can be hastened, and surfactant production set earlier in gestation, by the administration of steroids. Dr. Karlis Adamson, Associate Professor of Obstetrics, will be the operator in these experiments. Fetal rabbits will be obtained well ahead of the period of gestation when surfactant normally develops in this species (term - 3 days). The maternal abdominal cavity will be opened surgically, and the amniotic sac of individual fetuses in one horn will be injected with ACTH; the fetuses in the opposite horn will serve as operative shams and will receive an equal volume of vehicle. The abdominal incision will be closed, the mother will be permitted to recover, and the fetuses will be delivered 24 to 48 hours later by caesarian section. At that time, the lungs of the treated fetal animals will be studied in two ways: 1) to determine whether surface activity is precociously present in ACTH-injected fetuses compared with controls; 2) histological and histochemical studies of the maturing fetal lung will assess the activity of alkaline phosphatase enzyme, the presence of glycogen, and morphological differentiation. Alkaline phosphatase will be studied at the magnification of the electron microscope.

These experiments will be carried out as soon as Dr. Adamson feels comfortable with the rather tricky technique of injecting amniotic sacs without subsequent leakage of amniotic fluid.

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6. Budget Plan: †

† See complete budget sheet

| | | |
|---|---|---|
| a. | Salaries | $11,445 |
| b. | Expendable Supplies | 11,950 |
| c. | Other Expenses | 3,800 |
| d. | Permanent Equipment | 350 |
| e. | Overhead (15% of a, b, c) | 4,079 |
| | Total | $31,624 |

7. Anticipated Duration of Work:

3-5 years

8. Facilities and Staff Available: My laboratory has moved to new space in the William Black Research Building, where facilities for animal experimentation, including radioactive studies, are self-contained within the Department of Pediatrics (4th floor) research unit. Drs. Sommers and Goldenberg offer a completely equipped Department of Pathology at Francis Delafield Hospital, across the street. There are two assistants who both work on two interrelated research projects (vide infra). Mrs. Mildred Rothenberg is in charge of lab business in addition to her duties as senior technician. Mr. King, a junior technician, runs the surface balance and, in addition, is assistant in all animal experiments. He is gaining some chemical expertise under guidance.

9. Additional Requirements:

None contemplated

10. Additional Information (Including relation of work to other projects and other sources of support):
My own salary and that of my senior technician are made available through an NIH research grant entitled Amine Handling as a Factor in Newborn Adaptation (HD-00746-), presently beginning its fourth year of support. The motivation for the two projects was a single idea and, as data presently reveal (section 1 and 2), the projects are inextricably associated. The 1966 total NIH support (part of which goes to Drs. Sommers and McNary) for 1966 is $39,000. I plan to continue separate investigations under an NIH grant unrelated to lung surfactant and, in 1968, will apply for a 5-year renewal of NIH support.

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Signature Dr Dwight-MD
Director of Project

Business Officer of the Institution

Source: https://www.industrydocumentslibrary.ucsf.edu/tobacco/0006...

Budget 1966-1967

## A. Personnel

| | | |
|---|---|---|
| Surface balance, animal technician (Floyd King) | 100% | $4,800 |
| Histochemistry, radioautography technician (SCS) (Ruth Stover) | 50% | * 3,200 |
| Machinist, repair and construction, dept. share salary (George Salata) | | 1,000 |
| Dishwasher (Moses Clemons) | 10% | 500 |
| Typist at $3.00/hour (Mrs. V. Sommerick and Mrs. B. Mellenhoff) | | 1,000 |
| | | $10,500 |
| Fringe 9% | | 945 |
| Total | | $11,445 |

## B. Consumable Supplies

| | |
|---|---|
| Histochemical supplies, autography, etc. (SCS) | * $3,000 |
| Purchase radioactive chemicals (S-35, Palmitate, Thymidine) | 2,500 |
| Lab supplies, reagents, glassware, drugs, hyperbaric chamber fixtures, oxygen | 3,650 |
| 1/2 time maintenance electron microscope (SCS) | * 800 |
| Animal purchase | 2,000 |
| Total | $11,950 |

## C. Miscellaneous

| | |
|---|---|
| Reprints, slides, photos, photocopying supplies, books, telephone, stationery | $1,500 |
| Travel to one scientific meeting | 200 |
| Travel to and from Boston (experiments) | 300 |
| Color plates (Publication), 3 | 1,800 |
| Total | $3,800 |

## D. Permanent Equipment

| | |
|---|---|
| Photocopy machine (M M dry) | $350 |
| Total | $350 |

| | |
|---|---|
| 15% - A, B, C ($27,195) ..... | $4,079 |
| Grand Total ..... | $31,624 |

* Dr. S. C. Sommers.

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Source: https://www.industrydocuments.ucsf.edu/docs/tnwm0038